

**Title:**

**Mammalian prion propagation in PrP transgenic *Drosophila***

**One Sentence Summary:**

**Mammalian prion replication in *Drosophila***

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## Abstract:

Mammalian prions propagate by template-directed misfolding and aggregation of normal cellular prion related protein PrP<sup>C</sup> as it converts into disease-associated conformers collectively referred to as PrP<sup>Sc</sup>. Mammalian species may be permissive for prion disease because these hosts have co-evolved specific co-factors that assist PrP<sup>C</sup> conformational change and prion propagation. We have tested this hypothesis by examining whether faithful prion propagation occurs in the normally PrP<sup>C</sup>-null invertebrate host *Drosophila melanogaster*. Ovine PrP transgenic *Drosophila* exposed at the larval stage to ovine scrapie showed a progressive accumulation of transmissible prions in adult flies. Strikingly, the biological properties of distinct ovine prion strains were maintained during their propagation in *Drosophila*. Our observations show that the co-factors necessary for strain-specific prion propagation are not unique to mammalian species. Our studies establish *Drosophila* as a novel host for the study of transmissible mammalian prions.

## Key words:

PrP<sup>Sc</sup>, prion, infectivity, *Drosophila*, strains

## Abbreviations:

ARQ	A <sup>136</sup> R <sup>154</sup> Q <sup>171</sup> ovine PrP
BSE	Bovine Spongiform Encephalopathy
CJD	Creutzfeldt-Jakob disease
GPI	Glycosylphosphatidyl inositol
PI	Performance Index
PMCA	Protein misfolding cyclic amplification
QuIC	Quaking-induced conversion
VRQ	V <sup>136</sup> R <sup>154</sup> Q <sup>171</sup> ovine PrP

## Introduction

Mammalian prions cause fatal neurodegenerative diseases such as Creutzfeldt-Jakob disease (CJD) in humans, Bovine Spongiform Encephalopathy (BSE) in cattle and scrapie disease of sheep (Prusiner, 2004). These conditions are transmissible between individuals of the same or different species and as a consequence, animal prion diseases pose a realistic threat to human health through their zoonotic potential (Bruce et al., 1997). Prions lack a conventional nucleic acid-based genome. The ‘protein only’ concept that relates to prion diseases predicts that infectious prion particles consist of PrP<sup>Sc</sup> in the form of aggregates of misfolded conformers of the normal host protein PrP<sup>C</sup> (Prusiner, 1982). Prion propagation occurs by template-directed nucleation whereby a prion seed induces conformational change within PrP<sup>C</sup> and promotes the incorporation of nascent misfolded prion protein into growing PrP<sup>Sc</sup> assemblies, which subsequently undergo fragmentation (Collinge, 2016).

PrP<sup>C</sup> is a cell-surface glycoprotein attached to the membrane by a glycosylphosphatidyl inositol (GPI) anchor (Stahl et al., 1987). It is now well established that PrP<sup>C</sup> expression is obligatory for prion replication and prion-induced neurotoxicity (Bueler et al., 1993; Mallucci et al., 2003). A remarkable feature of mammalian prions is the existence of different strains of the transmissible agent that directly influence host range and the clinico-pathological features of prion disease in the affected host (Prusiner, 2004). According to the ‘protein only’ concept (Prusiner, 1982), prion strain-specific properties are dictated by the conformational arrangement of PrP<sup>Sc</sup> (Bessen and Marsh, 1994; Safar et al., 1998; Telling et al., 1996). Changes in the replication environment of prions can induce mutational change in their strain properties (Collinge and Clarke, 2007), an event that may occur during inter-species prion transmission where PrP primary structures differ (Bartz et al., 2000; Kimberlin et al., 1987).

Several fundamental features of mammalian prion biology remain unresolved despite considerable efforts aimed at their elucidation (Collinge, 2016). These include lack of a full description of the molecular and cellular machinery necessary to generate infectious neurotoxic prions (Supattapone, 2014). The possibility exists that mammalian hosts, in which PrP<sup>C</sup> is ubiquitously expressed and well conserved, are permissive for prion formation because they have co-evolved specific co-factors that assist in prion protein misfolding and propagation. Here we have tested this hypothesis by examining whether authentic prion propagation occurs in *Drosophila melanogaster*, an invertebrate species that is

95 phylogenetically separated from mammals by millions of years of divergent evolution.  
96 *Drosophila* and other members of the arthropod phylum do not show the presence of an  
97 orthologous PrP gene in their genome and therefore do not normally express PrP<sup>C</sup> (Rivera-  
98 Milla et al., 2006).

99  
100 In this study, we tested the capacity of mammalian prions to faithfully propagate in a strain-  
101 specific manner in *Drosophila* genetically engineered to express PrP<sup>C</sup> (Thackray et al., 2012b;  
102 Thackray et al., 2014). Exposure of larval stage PrP transgenic *Drosophila* to mammalian  
103 prions resulted in a progressive accumulation of *bona fide* prions in adult flies. Strikingly, the  
104 biological properties of the original prion strain used as inoculum were maintained during  
105 their passage in the insect host. These observations show for the first time that the molecular  
106 and cellular co-factors necessary for strain-specific prion propagation are conserved between  
107 phylogenetically diverse species and are not unique to mammalian hosts. Our studies provide  
108 a fundamental advance in the understanding of mammalian prion biology and also establish  
109 *Drosophila* as a novel methodology for the study of transmissible mammalian prions.



## Materials and Methods

### Fly stocks

The *UAS*-PrP fly lines w; M{VRQ-PrP(GPI), 3xP3-RFP.attP}ZH-51D and w; M{ARQ-PrP(GPI), 3xP3-RFP.attP}ZH-51D transgenic for ovine V<sup>136</sup>R<sup>154</sup>Q<sup>171</sup> (VRQ) or A<sup>136</sup>R<sup>154</sup>Q<sup>171</sup> (ARQ) PrP, respectively, expressed with an N-terminal leader peptide and C-terminal GPI signal sequence, were generated by PhiC31 site-specific transformation by Bestgene (California, USA) as previously described (Thackray et al., 2012c). The following fly lines were obtained from the Department of Genetics, University of Cambridge, UK.

*Actin-5C-GAL4* (y w; P{w[+mC]=Act5C-Gal4}25F01/CyO, y[+])

*Elav-GAL4* (P{w[+mW.hs]=GawB}elav[C155])

51D (w; M{3xP3-RFP.attP}ZH-51D)

*Cre*-mediated removal of the red fluorescent protein (RFP) gene from the VRQ and 51D fly genome was performed by conventional fly crosses (Thackray et al., 2014). PrP transgenic *Drosophila* were crossed with either the *Elav-GAL4* or *Actin-5C-GAL4* driver fly lines to derive transgenic flies that expressed PrP pan neuronally or ubiquitously, respectively. 51D *Drosophila* crossed with either driver fly line were used as control flies where appropriate. All fly lines were raised on standard cornmeal media at 25°C and maintained at low to medium density, and pre-mated before experimental use.

### Prion inoculation of *Drosophila*

*Primary transmission of sheep scrapie (sheep-to-fly):* *Drosophila* at the larval stage of development were exposed to brain homogenate of cerebral cortex tissue from a confirmed PG127 (alternatively referred to as DAW or G<sub>338</sub>) scrapie-positive sheep (SE1848/0005) (Thackray et al., 2008). New Zealand-derived VRQ/VRQ scrapie-free brain tissue was used as control material. Two hundred and fifty microlitres of a 1% (w/v) sheep brain homogenate prepared in PBS pH7.4, were added to the top of the cornmeal that contained third instar *Drosophila* larvae in 3-inch plastic vials. Following eclosion (i.e. hatching) flies were transferred to fresh non-treated vials. *Secondary transmission of sheep scrapie (fly-to-fly):* *Drosophila* head homogenates were prepared from 30 day old flies that had been exposed at the larval stage to scrapie-positive or scrapie-negative sheep brain material. Two hundred and fifty microlitres of a 10<sup>-1</sup> (v/v) dilution of the original fly brain homogenate were added to the top of the cornmeal that contained third instar *Drosophila* larvae in 3-inch plastic vials. Flies were transferred to fresh, non-treated vials following eclosion. *Transmission of defined ovine*

*prion strains in PrP transgenic Drosophila: Drosophila* at the larval stage of development were exposed to 1% (w/v) mouse brain homogenate infected with a defined ovine prion strain or prion-free mouse brain homogenate as control. The different mouse-adapted prion strains used here were isolated by serial passage of sheep scrapie isolates in ovine PrP transgenic mice as previously described (Thackray et al., 2011; Thackray et al., 2012a).

### **Preparation of *Drosophila* head homogenate**

Whole flies in an eppendorf tube were frozen in liquid nitrogen for 10 minutes and then vortexed for 2 minutes to cause decapitation. Individual fly heads were isolated and placed in clean eppendorf tubes using a fine paint brush. PBS pH7.4 was added to give 1µL / head and homogenates were prepared by manual grinding of the fly heads with sterilized plastic pestles. For western blot analysis, fly head homogenate was mixed with an equal volume of 20% scrapie-free sheep brain homogenate prior to extraction and PK digestion as previously described (Lacroux et al., 2012) using monoclonal antibody Sha31 (Feraudet et al., 2005).

### **Protein misfolding cyclic amplification (PMCA)**

PMCA was carried out as previously described (Lacroux et al., 2012). The substrate consisted of 10% (w/v) ovine VRQ PrP transgenic mouse (tg338) brain homogenate in PBS pH 7.4, 0.1% Triton X-100 and 150 mM NaCl buffer. Five µL of fly head homogenate were mixed with 45µL of substrate in 0.2 mL thin wall PCR tubes. Sealed tubes were then placed in the horn of a Misonix 4000 sonicator for one round of 96 cycles. Each cycle consisted of a 10 second sonication step (70% of power) followed by a 14 minute and 50 second incubation step. Twenty µL of each reaction mix were subsequently treated with PK (4µg of PK per mg of protein) at 37°C for 2 hours and the reaction stopped by adding Pefabloc (4mM final concentration). PK-resistant PrP was detected by western blot as previously described (Lacroux et al., 2012) using monoclonal antibody Sha31 (Feraudet et al., 2005).

### **Mouse prion bioassay**

Mouse prion bioassays were carried out in tg338 mice, which are transgenic for ovine VRQ PrP and are highly efficient for the detection of sheep scrapie infectivity (Le Dur et al., 2005). Mice (n=6 per inocula) were injected intracerebrally with 20µL of diluted fly head homogenate (to give approximately 2 fly head equivalents per mouse) and monitored daily until the occurrence of clinical signs of mouse prion disease. Inoculated mice were euthanized when they started to show locomotor disorders and any impairment in their capacity to feed,

or at a pre-defined end-point for the assay (either >250 days or in some cases >670 days post inoculation) (Andreoletti et al., 2011). Brain tissue (cerebral cortex) was collected from euthanized mice and frozen for PrP<sup>Sc</sup> analysis by Western blot (TeSeE, BioRad) or PET blot analysis (Andreoletti et al., 2011).

### **Negative geotaxis climbing assay**

The locomotor ability of flies was assessed in a negative geotaxis climbing assay initiated with 45 (3 x n=15) age-matched, pre-mated female flies in each treatment group. *Drosophila* were placed in adapted plastic 25mL pipettes that were used as vertical climbing columns and allowed to acclimatise for 30 minutes prior to assessment of their locomotor ability. Flies were tapped to the bottom of the pipette (using the same number and intensity of taps on each occasion) and then allowed to climb for 45 seconds. At the end of the climbing period the number of flies above the 25mL mark, the number below the 2mL mark and the number in between the 2mL and 25mL mark was recorded. This procedure was performed three times at each time point. The performance index (PI) was calculated for each group of 15 flies (average of 3 trials) using the formula:

$$PI = 0.5 \times (n_{total} + n_{top} - n_{bottom}) / n_{total}$$
where  $n_{total}$  is the total number of flies,  $n_{top}$  is the total number of flies at the top, and  $n_{bottom}$  is the total number of flies at the bottom. A PI value of 1 is recorded if all flies climb to the top of the tube whereas the value is 0 if no flies climb the tube past the 2mL mark. The mean PI  $\pm$  SD at individual time points for each treatment group was plotted as a regression line.

### **Statistical analysis**

Statistical analysis of the negative geotaxis climbing assay data was performed by the paired Student *t* test, using Prism (GraphPad Software Inc, San Diego, USA).

## Results

### Mammalian prions replicate in PrP transgenic *Drosophila*

Transgenic *Drosophila* that express membrane-bound VRQ ovine PrP (VRQ *Drosophila* fly line) and control non-transgenic flies (51D fly line) were exposed at the larval stage to brain material prepared from either PG127 scrapie-infected or healthy prion-free sheep (Thackray et al., 2012c; Thackray et al., 2014). After hatching, *Drosophila* were transferred to prion-free culture tubes. At various time points ( $\leq 40$  days) during their adult lifespan, groups of *Drosophila* were euthanized, decapitated and homogenate prepared from the isolated fly heads. These homogenates were used to seed *in vitro* PMCA reactions in order to reveal the presence of prion seeding activity as shown by the data in Figure 1. PMCA mimics prion replication *in vivo*, but in an accelerated form, allowing amplification of minute quantities of PrP<sup>Sc</sup> and prion infectivity (Saborio et al., 2001). In PMCA reactions, a PrP<sup>C</sup>-containing substrate (in this study brain homogenate prepared from ovine PrP transgenic mice) is combined with the seed (in this study *Drosophila* head homogenate) that may contain minute amounts of PrP<sup>Sc</sup>. Following repeated cycles of incubation and sonication, the amount of PrP<sup>Sc</sup> seed increases to levels at which it can be detected by conventional biochemical means, such as western blot detection of PK-resistant PrP<sup>Sc</sup> as was used here.

No prion seeding activity was detected in PMCA reactions with seed prepared from mock-infected VRQ *Drosophila* head homogenate, or from scrapie-exposed or mock-infected 51D control flies (Figure 1A). Similarly, no prion seeding activity was detected in head homogenates prepared from 5 or 10 day old scrapie-exposed VRQ *Drosophila*. Strikingly, prion seeding activity was detected in head homogenate prepared from scrapie-infected VRQ *Drosophila* aged  $\geq 20$  days. An end-point titration of prion seeding activity, carried out using serial 10-fold dilutions of original fly head homogenate, indicated a progressive increase of prion seeding titre in the heads of prion-exposed VRQ *Drosophila* from 20 to 40 days of age (Figure 1B). The seeding activity in 40 day old prion-exposed VRQ *Drosophila* was  $10^{-4}$  lower than that seen in a terminal disease-PG127 sheep scrapie inoculum (Thackray et al., 2016).

We determined if the presence of the prion seeding activity in scrapie-inoculated VRQ *Drosophila* was associated with the presence of PK-resistant PrP<sup>Sc</sup>, a pathognomonic marker

of prion disease in mammalian hosts (Prusiner, 2004). Head homogenate from 40 day old *Drosophila* was PK-digested and analyzed directly by SDS-PAGE and western blot probed with an anti-PrP monoclonal antibody. The data in Figure 2 show that no PK-resistant PrP<sup>Sc</sup> signal was detected in *Drosophila* exposed to scrapie-free sheep brain material (Figures 2A and 2B), or from scrapie-treated 51D flies (Figure 2C). Notably, a PK-resistant PrP signal was detected in head homogenate from scrapie-exposed VRQ *Drosophila* (Figure 2D). The molecular profile of the PK-resistant PrP in VRQ *Drosophila* was clearly different to that present in the original PG127 inoculum, an expected feature considering differences in N-linked glycosylation that exists between *Drosophila* and mammalian hosts (Choi et al., 2010; Thackray et al., 2012c). The intensity of the PK-resistant PrP signal in the VRQ *Drosophila* head homogenate was similar to that obtained in a 10<sup>-4</sup> dilution of a terminal disease-PG127 sheep scrapie inoculum. This result was consistent with the data obtained in the PMCA end point titration where 40 day old VRQ *Drosophila* displayed a 10<sup>-4</sup> lower seeding activity than a terminal disease-PG127 scrapie inoculum.

### **Fly-to-mouse prion transmission**

In order to unequivocally demonstrate that authentic prions replicated in scrapie-exposed VRQ *Drosophila* we performed fly-to-mouse transmission. Head homogenate from flies used as a seed in the PMCA experiment shown above was inoculated into ovine VRQ PrP transgenic (tg338) mice. The transmission data are shown in Table 1. No evidence of clinical prion disease or abnormal PrP accumulation were observed in mice inoculated with head homogenates prepared from VRQ *Drosophila* and 51D flies exposed to control sheep brain homogenate. Similarly, no prion infectivity was detected in tg338 mice inoculated with head homogenates prepared from 5 or 10 day old PG127-exposed VRQ *Drosophila*. An incomplete attack rate for clinical prion disease transmission was observed in tg338 mice inoculated with head homogenate from 20 day old scrapie-infected VRQ *Drosophila*. Mice inoculated with head homogenates prepared from 30 and 40 day old scrapie-exposed VRQ *Drosophila* developed a 100% attack rate for clinical prion disease transmission. The brains of tg338 mice inoculated with fly-head homogenate were probed for disease-associated PrP as shown by the data in Figure 3. Prion disease in mice that showed clinical signs of the condition was confirmed by western blot detection of PK-resistant PrP<sup>Sc</sup> (Figure 3A). PET blot analysis of the brains of clinically affected mice showed the typical distribution of PrP<sup>Sc</sup> in PG127 scrapie-inoculated tg338 mice (Figure 3B). No clinical signs of mouse prion disease or abnormal PrP accumulation were observed in tg338 mice inoculated with scrapie-exposed

51D fly head homogenate. This result excludes the suggestion that carry-over of the original sheep inoculum was an explanation for prion infectivity detection in scrapie-exposed VRQ *Drosophila*.

To further increase the robustness of this observation, the fly-to-mouse transmission experiment was repeated twice using samples obtained in two independent experiments (using the same fly lines and the same sheep scrapie prion strain). Supplementary Data S1 shows that the observed incubation times and attack rates in these repeat experiments were consistent to those recorded in the original experiment. Collectively these data unequivocally demonstrate that ovine prion infectivity progressively accumulated in scrapie-exposed ovine VRQ PrP transgenic *Drosophila*.

### **Fly-to-Fly prion propagation**

We next investigated whether the prion infectivity that accumulates in VRQ *Drosophila* at first passage could be serially propagated in the same fly line. Accordingly, head homogenate from 30 day old adult *Drosophila* (first passage flies) was used to inoculate fresh VRQ *Drosophila* (second passage flies) at the larval stage. Second passage VRQ *Drosophila* were allowed to hatch and groups of flies were euthanized at 5, 30 and 40 days of age when head homogenate was prepared for PMCA analysis. The data in Supplementary Data S2 show, as expected, no PMCA prion seeding activity was detected in VRQ *Drosophila* that were exposed to head homogenate from first passage mock-infected VRQ *Drosophila*, or first passage scrapie- or mock infected 51D flies. However, prion seeding activity was detected in second passage VRQ *Drosophila* exposed to 30 and 40 day old, but not 5 day old, head homogenate from first passage scrapie-exposed VRQ *Drosophila*. Endpoint titration of the PMCA-positive second passage VRQ *Drosophila* samples indicated an increase in prion seeding activity titre in these flies between 30 and 40 days of age.

Second passage VRQ *Drosophila* head homogenate was inoculated into tg338 mice in order to assess prion infectivity in these samples. Mice that received 30 day old PMCA-positive second passage VRQ *Drosophila* head homogenate showed 100% attack rate for clinical signs of mouse prion disease and an incubation period of  $89 \pm 4$  days. The brains of inoculated mice were examined for the presence of disease-associated PrP as shown by the data in Supplementary Data S3. PK-resistant PrP<sup>Sc</sup> was evidenced by western blot of the brains of clinically affected mice and PET blot analysis of the brains of clinically affected mice

revealed the typical distribution of PrP<sup>Sc</sup> in PG127 scrapie-inoculated tg338 mice. No clinical signs or abnormal PrP accumulation was observed in tg338 mice inoculated with 5 day old second passage VRQ *Drosophila* head homogenate or from VRQ *Drosophila* exposed to first passage 51D control flies. These results demonstrate that mammalian prions propagated in VRQ *Drosophila* can be serially transmitted in flies.

### **Prion-induced toxic phenotype in PrP transgenic *Drosophila***

Since prion-induced toxicity occurs concomitantly with prion replication in mammalian hosts (Bueler et al., 1993; Mallucci et al., 2003) we next investigated whether the propagation of prions in VRQ *Drosophila* induced a toxic phenotype in these flies. To do so, we performed a negative geotaxis climbing assay (Thackray et al., 2012c) using adult *Drosophila* previously exposed at the larval stage to ovine scrapie prions. The data in Supplementary S4 (together with the accompanying statistical analysis described in the Supplementary Materials for this figure) show that there was no difference in the climbing ability between 51D flies exposed to PG127 scrapie or control prion-free sheep brain homogenate. Strikingly, VRQ *Drosophila* developed a toxic phenotype after exposure to PG127 prions, evidenced by an accelerated decrease in locomotor ability compared to control treated flies, that became progressively more severe with age. Collectively, these data show that the prion-induced toxic fly phenotype was associated with prion propagation in adult PrP transgenic *Drosophila* as evidenced by the data in Table 1 and Figure 3.

### **Prion strain properties are maintained in PrP transgenic *Drosophila***

A comparison of the incubation period and PK-resistant PrP<sup>Sc</sup> molecular profile observed in tg338 mice inoculated with either a sample of the original PG127 sheep scrapie inoculum, or this material passaged in VRQ *Drosophila*, suggested that mammalian prion strain properties were unaltered following propagation in PrP transgenic *Drosophila*. To confirm this was the case we propagated three distinct ovine prion strains in PrP transgenic *Drosophila* prior to their re-isolation in tg338 mice. The panel of ovine prion strains used here have been previously characterized by bioassay in tg338 mice and are differentiated by unique incubation times and on the basis of their PK-resistant PrP<sup>Sc</sup> western blot molecular profile (Thackray et al., 2011; Thackray et al., 2012a). We exposed ovine PrP transgenic *Drosophila* at the larval stage to each ovine scrapie prion strain. VRQ *Drosophila* were exposed to ovine prions isolated in VRQ PrP transgenic mice and ARQ *Drosophila* were exposed to ovine prions isolated in ARQ PrP transgenic mice. Collectively, the ovine PrP transgenic

*Drosophila* were susceptible to all three different prion strains shown by the development of a progressive toxic phenotype in the form of an accelerated decline in performance in a negative geotaxis climbing assay (see Supplementary Data S4).

An aliquot of each original prion strain isolated in PrP transgenic mice and samples of these prions propagated in PrP transgenic *Drosophila* were transmitted to tg338 mice (for either one or two passages). The attack rate and incubation period observed in tg338 mice inoculated with the original prion strains were similar to those obtained following inoculation with the equivalent prion strain propagated in PrP transgenic *Drosophila* as shown by the data in Table 2. The brains of clinically prion-diseased tg338 mice were examined for disease-associated PrP and neuropathology as shown by the data in Figure 4. Irrespective of the number of passages, the molecular profile of PK-resistant PrP<sup>Sc</sup> observed in the brains of tg338 mice inoculated with the original ovine prions and those propagated in PrP transgenic *Drosophila* were indistinguishable (Figure 4A). In addition, the vacuolar lesion profile in the brains of tg338 mice inoculated with the original ovine prion strain and with the equivalent prion strain propagated in PrP transgenic *Drosophila* were virtually identical (Figure 4B). Collectively, these data demonstrate that the biological properties of distinct ovine prion strains were maintained after their propagation in ovine PrP transgenic *Drosophila*.



## Discussion

In our study presented here we have demonstrated that scrapie-exposed *Drosophila* transgenic for ovine PrP expressed pan neuronally displayed a progressive accumulation of prion seeding activity and showed the presence of PK-resistant PrP. Crucially, we also demonstrated a progressive accumulation of prion infectivity in these *Drosophila* by bioassay in tg338 mice. These features unequivocally demonstrate for the first time the replication of *bona fide* mammalian prions in a PrP transgenic invertebrate host.

The central role of PrP in prion replication has been verified by infectivity studies in mammalian hosts, principally mice, with either gene knock-out or transgenic over-expression of endogenous prion protein expression (Brandner and Jaunmuktane, 2017). These extensive studies have collectively established that mammalian prions do not replicate in hosts that fail to express PrP<sup>C</sup>. However, several lines of evidence have suggested that expression of PrP<sup>C</sup> alone is not sufficient to confer susceptibility to prion propagation (Courageot et al., 2008; Enari et al., 2001; Raeber et al., 1999). This has led to the suggestion that specific molecular co-factors and/or cellular machinery are crucial for prion propagation. The nature and function of co-factors in prion propagation remains undefined (Ma, 2012), although their role is highlighted by *in vitro* reconstitution experiments. To date, RNA or lipid has been shown to be required for the generation of prions with high infectious titre (Deleault et al., 2003; Deleault et al., 2012; Wang et al., 2010). However, it is possible that other molecular components participate in the process of prion replication and the definitive list of these co-factors and their mode of action remain to be established (Castilla et al., 2008). Insects are invertebrate hosts that are phylogenetically separated in evolutionary terms from mammals by millions of years. We have demonstrated here that *Drosophila*, a normally PrP-null insect host, rendered transgenic solely for mammalian PrP, are permissive for the faithful replication of a panel of three mammalian prion strains. This provides a cogent argument that the nature of the molecular and cellular factors that facilitate prion replication do not result from a co-evolutionary selection process between the infectious agent and their natural hosts.

An intriguing feature in mammalian prion biology is the existence of prion strains. These are identified as different prion isolates from individuals of the same species that produce distinct disease phenotypes upon serial passage in isogenic hosts (Bruce and Dickinson, 1987). Glycosylation is an important factor in the determination and maintenance of conformation,

function and interactions of glycoproteins (O'Connor and Imperiali, 1996). Mammalian PrP expressed in the natural host has two N-linked carbohydrate moieties attached to asparagine residues located in the C-terminal domain of the protein (Endo et al., 1989). Transmission experiments carried out in mice transgenic for PrP devoid of either one or both N-linked carbohydrate moieties, as a result of changes to the prion protein primary structure, indicated an alteration in the biological properties of some of the prion strains tested (Cancellotti et al., 2013). These results supported the view that N-linked glycosylation of PrP<sup>C</sup> could participate in the transfer and/or maintenance of the prion strain-specific information. Mammalian N-linked glycoproteins, including those attached to PrP<sup>C</sup> expressed in the natural host, typically comprise complex glycans that consist of N-acetylglucosamine (GlcNAc), mannose, galactose, and terminal sialic acid residues (Endo et al., 1989). In contrast, the majority of neurones in the *Drosophila* brain synthesise N-linked glycans with core structures similar or identical to those produced by all eukaryotes but fail to acquire complex carbohydrate structures (März et al., 1995). Our study here demonstrates that the biological properties of distinct ovine prion strains were maintained after propagation in PrP transgenic *Drosophila*. Therefore, differences in N-linked glycosylation moieties between mammalian and insect species had no apparent impact on the biological properties of the different prion strains that we propagated in PrP transgenic *Drosophila*.

We have shown that VRQ *Drosophila* exposed to scrapie prions at the larval stage show an accelerated decline in locomotor ability at adulthood (Thackray et al., 2012b; Thackray et al., 2014; Thackray et al., 2016). The severity of this prion-induced toxic fly phenotype increased as the flies aged and correlates with the accumulation of prion seeding activity and infectivity in PrP transgenic *Drosophila*. In mammalian species clinical signs in prion affected individuals arise as a consequence of neurodegenerative processes initiated by the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>. Only cells that express PrP<sup>C</sup> are susceptible to prion-induced toxicity and formation of the toxic moiety occurs concomitantly with prion replication (Bueler et al., 1993; Mallucci et al., 2003). Despite significant advances (Moreno et al., 2012), the mechanisms responsible for neurodegeneration in prion disease and the role of PrP<sup>C</sup> and PrP<sup>Sc</sup> remain incompletely defined. Unravelling events that are directly triggered by prion protein conversion from those arising as a consequence of the perturbation of the central nervous system micro-environment is extremely challenging. PrP was expressed pan neuronally in the VRQ PrP transgenic fly line used to assess prion seeding activity and prion infectivity in the scrapie-infected *Drosophila* we have used here. We have previously shown that a prion-

induced toxic phenotype occurs in *Drosophila* transgenic for pan neuronal expression of PrP (Thackray et al., 2014; Thackray et al., 2016). Consequently, we consider that the prion-induced decline of the locomotor activity observed in PrP transgenic *Drosophila* reflects a neurotoxic phenotype that arises as a consequence of prion replication. This supports the view that PrP transgenic *Drosophila* can serve as a tractable system to probe for genetic modifiers of prion-induced neurodegeneration. *Drosophila* are increasingly used as a model system in the study of mammalian neurodegenerative disease including the pathobiology of Alzheimer's disease, Parkinson's disease and tauopathies (Lu and Vogel, 2009). This arises because the brains of *Drosophila* and mammalian species are composed of similar components (i.e. neurons and neuronal circuitry), and the nature of ion channels, neurotransmitters and synaptic proteins are highly conserved between mammals and the fly (Lessing and Bonini, 2009). Our use of PrP transgenic *Drosophila* to recapitulate mammalian prion infection provides an unprecedented opportunity to unravel the mechanisms of prion-induced neurotoxicity in a new animal system. *Drosophila* have already proved to be an important animal model to analyse the genetics of mammalian neurodegeneration. For example, resultant behavioural defects in *Drosophila* that arise through the deletion of a fly homolog of a human neurodegenerative gene can be compensated for by a functional human homologous gene (Luo et al., 1992). In addition, mutagenesis screens in *Drosophila* have led to the isolation of flies that show late-onset progressive degeneration of the adult nervous system that resembles human diseases (Min and Benzer, 1999).

Prion bioassays, which are generally carried out in conventional or transgenic mice, or primates or small ruminants, continue to provide a central role in the study of mammalian prion diseases. However, because of ethical and economic reasons, such as the cost of maintaining the vertebrate species during long bioassays, there is increasing pressure to limit their use and adopt alternative approaches to assess mammalian prion infectivity. Over the last decade, *in vivo* prion biology studies have been supplemented by *in vitro* techniques such as PMCA (Saa et al., 2005) and quaking-induced conversion (QuIC) (Atarashi et al., 2007). Both of these methodologies have been used for the detection of prion seeding activity, a biochemically and biologically relevant surrogate marker of prion infectivity. In this context, PMCA and QuIC provide rapid and sensitive quantitative detection of prion-infected samples. However, *in vitro* amplification methods remain refractory to many questions and experimental needs that require to be addressed in prion research. These methods are also obviously not adapted for investigating prion-induced neurodegeneration. *Drosophila* have

several important positive experimental advantages as an animal model to study neurodegenerative disease. Large numbers of flies can be produced in a short time, which correlated with their relatively short life span and simple genetics, allow rapid, statistically robust data collection. The ability of *Drosophila* to faithfully propagate mammalian prion strains has multiple potential applications. In relation to prion diagnostics, our *Drosophila*-based prion bioassay has already been shown to be capable of the sensitive detection of prion infectivity in brain tissue (Thackray et al., 2012b; Thackray et al., 2016) and blood (Thackray et al., 2016). PrP transgenic *Drosophila* could also help to address the need for new animal models that allow a relevant, rapid, robust and reasonably high throughput screening of therapeutic compounds against prion disease.

In our studies presented here we have demonstrated that core features of transmissible mammalian prion disease can be re-capitulated in PrP transgenic *Drosophila*. This opens a new era for the investigation of prion-induced neurotoxicity and prion replication mechanisms in a genetically well-defined tractable animal system. This new invertebrate model of mammalian prion disease will provide the opportunity to exploit the power of genetics in the fly to identify potential genetic modifiers of prion-induced neurotoxicity. Such genetic modifiers may serve as candidate diagnostic markers or therapeutic targets of human prion disease and prion-like diseases. In addition, the ease of transgenesis in *Drosophila* will allow the development of fly lines that express different species forms of PrP, such as bovine, cervid and human PrP to bioassay prion infectivity from these different mammalian hosts. For appropriate biosecurity, these future studies will require the BSE, CWD or CJD-inoculated *Drosophila* to be maintained at containment level 3 for prion research, an enhanced level of safety above the containment level 2 used for the sheep scrapie studies described here. In this manner, PrP transgenic *Drosophila* can be used to begin to address important questions on the pathogenic potential of known and possible zoonotic prions.

## Legends

### **Figure 1. Prion seeding activity in scrapie-exposed PrP transgenic *Drosophila***

*Elav* x VRQ(GPI) PrP transgenic (VRQ) and *Elav* x 51D (51D) *Drosophila* were exposed at the larval stage to PG127 scrapie-infected or prion-free control sheep brain material. At various times after hatching, head homogenate was prepared from harvested flies and used as seed in PMCA reactions. **(A)** End-point titration of PMCA prion seeding activity in head homogenate from control or PG127-exposed *Drosophila*; **(B)** Western blot detection of PK-resistant PrP<sup>27–30</sup> in PMCA reaction products seeded with control or PG127-exposed *Drosophila* head homogenate. Molecular mass markers in kDa shown on the left.

### **Figure 2. PK-resistant PrP in scrapie-exposed PrP transgenic *Drosophila***

*Elav* x VRQ(GPI) PrP transgenic (VRQ) and *Elav* x 51D (51D) *Drosophila* were exposed at the larval stage to PG127 scrapie-infected or prion-free control sheep brain material. At 30 days post-hatching, head homogenate was prepared from harvested flies, treated with PK and analysed by western blot to detect PK-resistant PrP. **(A)** and **(B)** *Drosophila* exposed to control brain homogenate; **(C)** and **(D)** *Drosophila* exposed to PG127 scrapie-infected sheep brain homogenate; **(A)** and **(C)** *Elav* x 51D *Drosophila*; **(B)** and **(D)** *Elav* x VRQ(GPI) PrP transgenic *Drosophila*. A titration of original PG127 scrapie-infected sheep brain homogenate is included on each blot for comparative purposes. Molecular mass markers in kDa shown in the middle of the Figure.

### **Table 1. Prion infectivity accumulates in scrapie-exposed PrP transgenic**

#### ***Drosophila***

*Elav* x VRQ(GPI) PrP transgenic (VRQ) and *Elav* x 51D (51D) *Drosophila* were exposed at the larval stage to PG127 scrapie-infected or prion-free control sheep brain material. At various times after hatching, head homogenate was prepared from harvested flies and intracerebrally inoculated into ovine PrP transgenic (tg338) mice. Inoculated mice were euthanized when they showed clinical signs of prion infection or after 250 days for those that did not develop clinical disease. Mice were considered positive for prion disease when PK-resistant PrP<sup>27–30</sup> was detected in brain tissue by western blot. The attack rate (number of prion positive mice/total number of mice inoculated) is reported for each treatment group. The

incubation period (IP) for inoculated mice, which represents the average time from inoculation to euthanasia for each inoculated group of animals, is reported in days  $\pm$  SD.

### **Figure 3. Detection of prion infectivity in scrapie-exposed PrP transgenic**

#### ***Drosophila***

*Elav* x VRQ(GPI) PrP transgenic (VRQ) and *Elav* x 51D (51D) *Drosophila* were exposed at the larval stage to PG127 scrapie-infected or prion-free control sheep brain material. At various times after hatching, head homogenate was prepared from harvested flies and inoculated into tg338 mice. Inoculated mice were euthanized when they showed clinical signs of prion infection or after 250 days for those that did not develop clinical disease. Mice were considered positive for prion disease when PK-resistant PrP<sup>27–30</sup> was detected in brain tissue by western blot. (A) Western blot detection of PK-resistant PrP<sup>27–30</sup> in the brains of tg338 mice with clinical prion disease. Molecular mass markers in kDa shown on the right; (B) PET blot analysis of tg338 mouse brains from animals inoculated with 40 day old *Elav* x VRQ(GPI) PrP transgenic (VRQ) or *Elav* x 51D (51D) *Drosophila* exposed to PG127 scrapie-infected or prion-free control sheep brain material. Scale bar represents 150  $\mu$ m.

### **Table 2: Scrapie strains retain transmission properties after passage in PrP transgenic *Drosophila***

The ovine classical scrapie prion strains PG127 and Apl<sub>338</sub> (isolated in ovine VRQ tg338 mice) and Pa<sub>59</sub> (isolated in ovine ARQ tg59 mice) were transmitted to tg338 ovine PrP transgenic mice. The ovine classical scrapie prion strains PG127, Pa<sub>59</sub> or Apl<sub>338</sub> were transmitted to tg338 ovine PrP transgenic mice. In parallel, *Actin* x VRQ(GPI) PrP transgenic (VRQ) *Drosophila* were exposed to the PG127 and Apl<sub>338</sub> prion strains, and *Elav* x ARQ(GPI) PrP transgenic (ARQ) *Drosophila* were exposed to the Pa<sub>59</sub> prion strain, at the larval stage. Head homogenates were prepared from adult flies aged 30 days and serially transmitted in tg338 mice (two iterative passages) by intracerebral inoculation. Mice were euthanized when they showed clinical signs of prion infection and after 250 days for those that did not develop clinical disease. Mice were considered positive for prion disease when PK-resistant PrP<sup>27–30</sup> was detected in brain tissue by western blot. The attack rate (number of prion positive mice/total number of mice inoculated) is reported for each treatment group. The incubation period (IP) for inoculated mice, which represents the mean time from

inoculation to euthanasia for each inoculated group of animals, is reported in days  $\pm$  SD. (NA: data not available, still ongoing).

#### **Figure 4. Authentic prion replication in PrP transgenic *Drosophila***

*Actin* x VRQ(GPI) PrP transgenic (VRQ) *Drosophila* were exposed to the PG127 and Apl<sub>338</sub>, prion strains, and *Elav* x ARQ(GPI) PrP transgenic (ARQ) *Drosophila* were exposed to the Pa<sub>59</sub> prion strain, at the larval stage. *Actin* x 51D and *Elav* x 51D (both referred to as 51D in appropriate graph) *Drosophila* were used as control flies where appropriate. Head homogenates were prepared from adult flies aged 30 days and inoculated into tg338 mice. Inoculated mice were euthanized when they showed clinical signs of prion infection or after 250 days for those that did not develop clinical disease. Mice were considered positive for prion disease when PK-resistant PrP<sub>27–30</sub> was detected in brain tissue by western blot. Mouse brains were analyzed for the presence of PK-resistant PrP and subjected to neuropathological assessment by PET blot and lesion profile analysis. **(A)** Western blot detection of PK-resistant PrP<sub>27–30</sub> in the brains of tg338 mice during the isolation of PG127, Pa<sub>59</sub> or Apl<sub>338</sub>, or prion strains (original inoculum), or these prion strains after passage in *Drosophila* (*Drosophila* passaged). Molecular mass markers in kDa are shown on the left; **(B)** PET blot and lesion profile analysis of tg338 mouse brains after exposure to the original mouse-derived PG127, Pa<sub>59</sub> or Apl<sub>338</sub>, or prion strains (original inoculum), or these prion strains after passage in *Drosophila* (*Drosophila* passaged). Scale bar represents 150  $\mu$ m.

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## **Supplementary Materials: Mammalian prion propagation in PrP transgenic *Drosophila***

### **Introduction**

We have successfully demonstrated that transmissible prions replicate in scrapie-exposed ovine PrP transgenic *Drosophila* (see main paper). In order to establish the robustness of our observations we performed repeat fly-to-mouse transmission experiments. We also investigated whether the prions that replicate in scrapie-exposed *Drosophila* were serially transmissible in the same fly line. In addition, we investigated whether prion replication in the fly was associated with prion-induced toxic phenotype as assessed by a negative geotaxis climbing assay.

### **Results and Discussion**

The data in Supplementary S1 show the attack rate (number of prion positive mice/total number of mice inoculated) and incubation period (IP) for repeat fly-mouse prion transmission experiments were similar to those observed in the original experiment shown in Table 1 of the main text. Collectively these data unequivocally demonstrate that ovine prion infectivity progressively accumulated in scrapie-exposed ovine VRQ PrP transgenic *Drosophila*.

The data in Supplementary S2 show that the prion seeding activity that accumulates in scrapie-exposed VRQ *Drosophila* at first passage could be serially propagated in the same fly line. No PMCA prion seeding activity was detected in VRQ *Drosophila* exposed to head homogenate from first passage mock-infected VRQ *Drosophila*, or first passage scrapie- or mock infected 51D flies. Prion seeding activity was detected in second passage VRQ *Drosophila* exposed to 30 and 40 day old, but not 5 day old, head homogenate from first passage scrapie-exposed VRQ *Drosophila*. Endpoint titration of the PMCA-positive second passage VRQ *Drosophila* samples indicated an increase in prion seeding activity titre in these flies between 30 and 40 days of age. These data demonstrate fly-to-fly prion propagation.

Second passage VRQ *Drosophila* head homogenate was inoculated into tg338 mice in order to assess the level of prion infectivity in these samples. Mice that received 30 day old PMCA-positive second passage VRQ *Drosophila* head homogenate showed 100% attack rate for



clinical signs of mouse prion disease and an incubation period of  $89 \pm 4$  days. The brains of inoculated mice were examined for the presence of disease-associated PrP as shown by Supplementary S3. PK-resistant PrP<sup>Sc</sup> was evidenced by western blot in the brains of clinically affected mice (Supplementary S3A). PET blot analysis of the brains of clinically affected mice showed the typical distribution of PrP<sup>Sc</sup> in PG127 scrapie-inoculated tg338 mice (Supplementary S3B). No clinical signs or abnormal PrP accumulation was observed in tg338 mice inoculated with 5 day old positive second passage VRQ *Drosophila* head homogenate or from VRQ *Drosophila* exposed to first passage 51D control flies. These results demonstrate that mammalian prions propagated in VRQ *Drosophila* can be serially transmitted in flies.

We investigated whether prion replication in scrapie-exposed PrP transgenic *Drosophila* induced a toxic phenotype in these flies. To do so, PrP transgenic *Drosophila* were exposed at the larval stage to prion-infected or prion-free tg338 or tg59 mouse brain homogenate. After hatching, the locomotor ability of prion-exposed and control *Drosophila*, expressed as a performance index, was assessed by a negative geotaxis climbing assay (Thackray et al., 2012c). The data in Supplementary S4 show the locomotor ability of adult *Drosophila* after exposure to ovine prions at the larval stage. Supplementary S4A shows that VRQ *Drosophila* displayed an accelerated decline in locomotor activity after exposure to both Apl<sub>338</sub> and PG127 prion strains, which was significantly different to the response seen by that of similar flies exposed to scrapie-free tg338 mouse brain homogenate ( $p=0.0001$ , and  $p=0.0008$ , respectively, assessed over days 1 - 40). The data in Supplementary S4B show that the locomotor ability of ARQ *Drosophila* after exposure to Pa<sub>59</sub> prions displayed a decline in locomotor activity that was significantly different to the response seen by that of similar flies exposed to scrapie-free mouse brain homogenate ( $p=0.0006$ , assessed over days 1 - 40). In contrast to these data, Supplementary S4C shows that control 51D flies displayed no difference in their locomotor ability after exposure to prion-infected or scrapie-free mouse brain homogenate ( $p>0.05$  in all cases, except Apl<sub>338</sub>  $p=0.0227$ , assessed over days 1 - 40). Collectively, these data show that PrP transgenic *Drosophila* are sensitive to prion-induced toxicity mediated by different prion strains.

## Supplementary Data Legends

### Supplementary S1: Reproducible fly-to-mouse transmission of prions from scrapie-exposed PrP transgenic *Drosophila*

*Elav* x VRQ(GPI) PrP transgenic (VRQ) and *Elav* x 51D (51D) *Drosophila* were exposed at the larval stage to PG127 scrapie-infected or prion-free control sheep brain material. At 30 days post hatching, head homogenate was prepared from harvested flies and inoculated into tg338 mice. Mice were euthanized when they showed clinical signs of prion infection and after 250 or 670 days post-inoculation for those that did not develop clinical disease. Mice were considered positive for prion disease when PK-resistant PrP27–30 was detected in brain tissue by western blot. The attack rate (number of prion positive mice/total number of mice inoculated) is reported for each treatment group. The incubation period (IP) for inoculated mice, which represents the average time from inoculation to euthanasia for each inoculated group of animals, is reported in days  $\pm$  SD.

### Supplementary S2. Serial transmission of prion seeding activity in scrapie-exposed PrP transgenic *Drosophila*

PG127 scrapie-infected or control prion-free sheep material was passaged in *Elav* x VRQ(GPI) PrP transgenic (VRQ) and *Elav* x 51D (51D) *Drosophila* (primary passage) and subsequently in *Elav* x VRQ(GPI) PrP transgenic (VRQ) flies (secondary passage). At various times after hatching, head homogenate was prepared from harvested flies and used as seed in PMCA reactions. **(A)** End-point titration of PMCA prion seeding activity in head homogenate from control or PG127-exposed *Drosophila*; **(B)** western blot analysis of PK-resistant PrP27–30 in PMCA reaction products seeded with *Drosophila* head homogenate. Molecular mass markers in kDa are shown on the left.

### Supplementary S3. Serial transmission of prion infectivity in scrapie-exposed PrP transgenic *Drosophila*

PG127 scrapie-infected or control prion-free sheep material was passaged in *Elav* x VRQ(GPI) PrP transgenic (VRQ) and *Elav* x 51D (51D) *Drosophila* (primary passage) and subsequently in *Elav* x VRQ(GPI) PrP transgenic (VRQ) flies (secondary passage). Head homogenate from second passage *Elav* x VRQ(GPI) PrP transgenic (VRQ) *Drosophila* was inoculated into tg338 mice. Inoculated mice were euthanized when they showed clinical signs

of prion infection or after 250 days for those that did not develop clinical disease. Mice were considered positive for prion disease when PK-resistant PrP27–30 was detected in brain tissue by western blot. **(A)** Western blot detection of PK-resistant PrP27–30 in the brains of tg338 mice with clinical prion disease. Molecular mass markers in kDa are shown on the right; **(B)** PET blot analysis of the brains from tg338 mice inoculated with 30 day old 2<sup>nd</sup> passage *Elav* x VRQ(GPI) PrP transgenic (VRQ) *Drosophila*. Scale bar represents 150  $\mu$ m.

#### **Supplementary S4. Prion-induced toxicity in scrapie-exposed PrP transgenic *Drosophila***

*Drosophila* were assessed for their locomotor ability by a negative geotaxis climbing assay following exposure at the larval stage to PG127 (red line), Apl<sub>338</sub> (light blue line); Pa<sub>59</sub> (green line) prion-infected, or prion-free tg338 (grey line) or tg59 (black line) ovine PrP transgenic mouse brain homogenate. The data shown are linear regression plots of the mean performance index  $\pm$  SD for three groups of flies per time point calculated as described in the Materials and Methods. *p* values assessed over days 1 – 40 of the climbing assay are shown on individual graphs. **(A)** *Actin* x VRQ(GPI) PrP transgenic (VRQ) *Drosophila*; **(B)** *Elav* x ARQ(GPI) PrP transgenic (ARQ) *Drosophila*; **(C)** *Actin* x 51D (green and black lines) and *Elav* x 51D (blue, red and grey lines).

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856



Figure 1. Prion seeding activity in scrapie-exposed PrP transgenic *Drosophila*

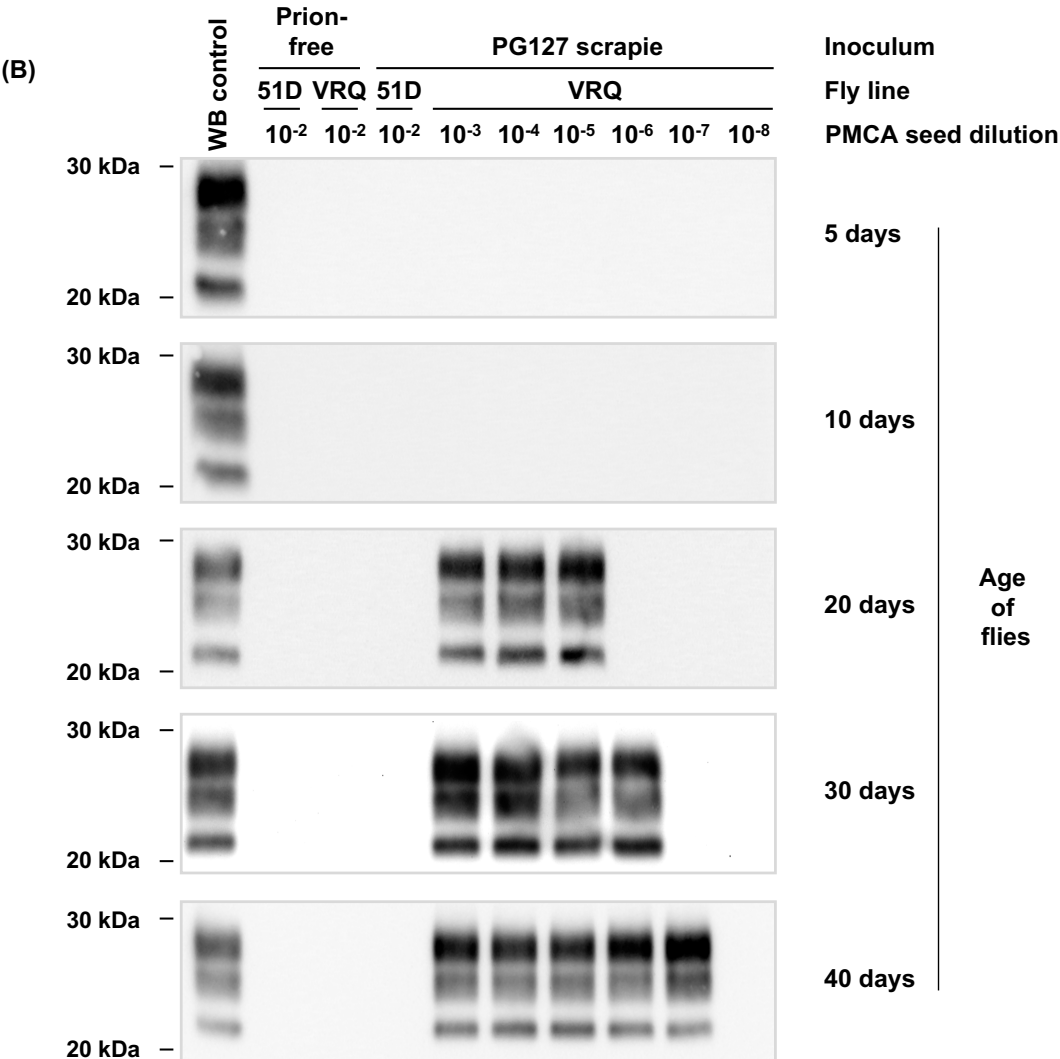
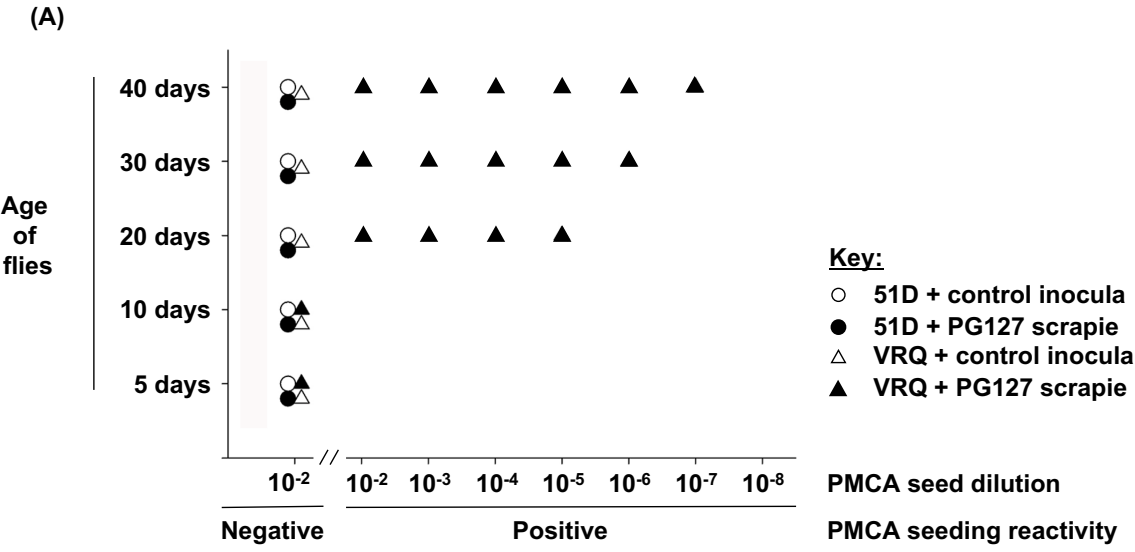
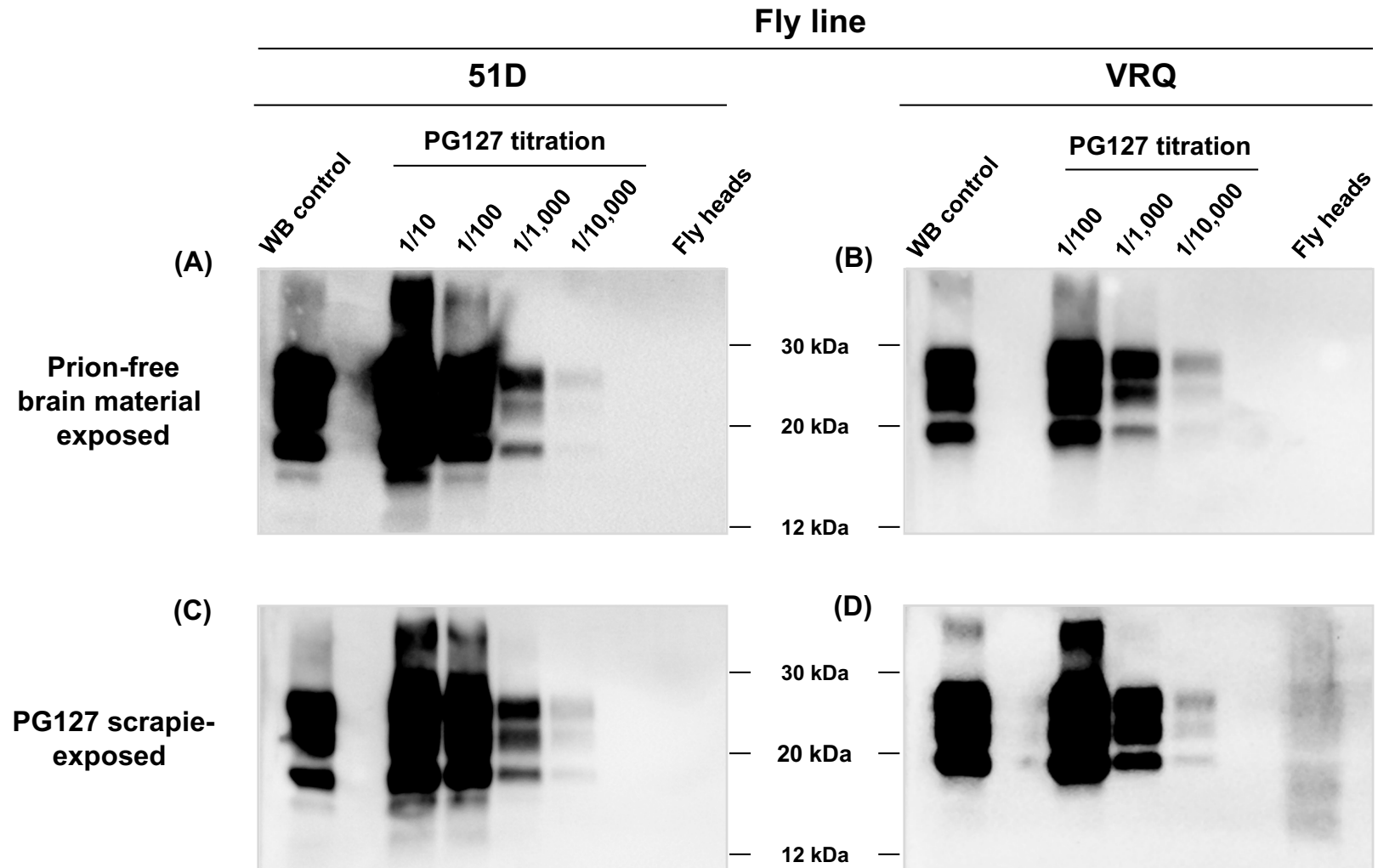


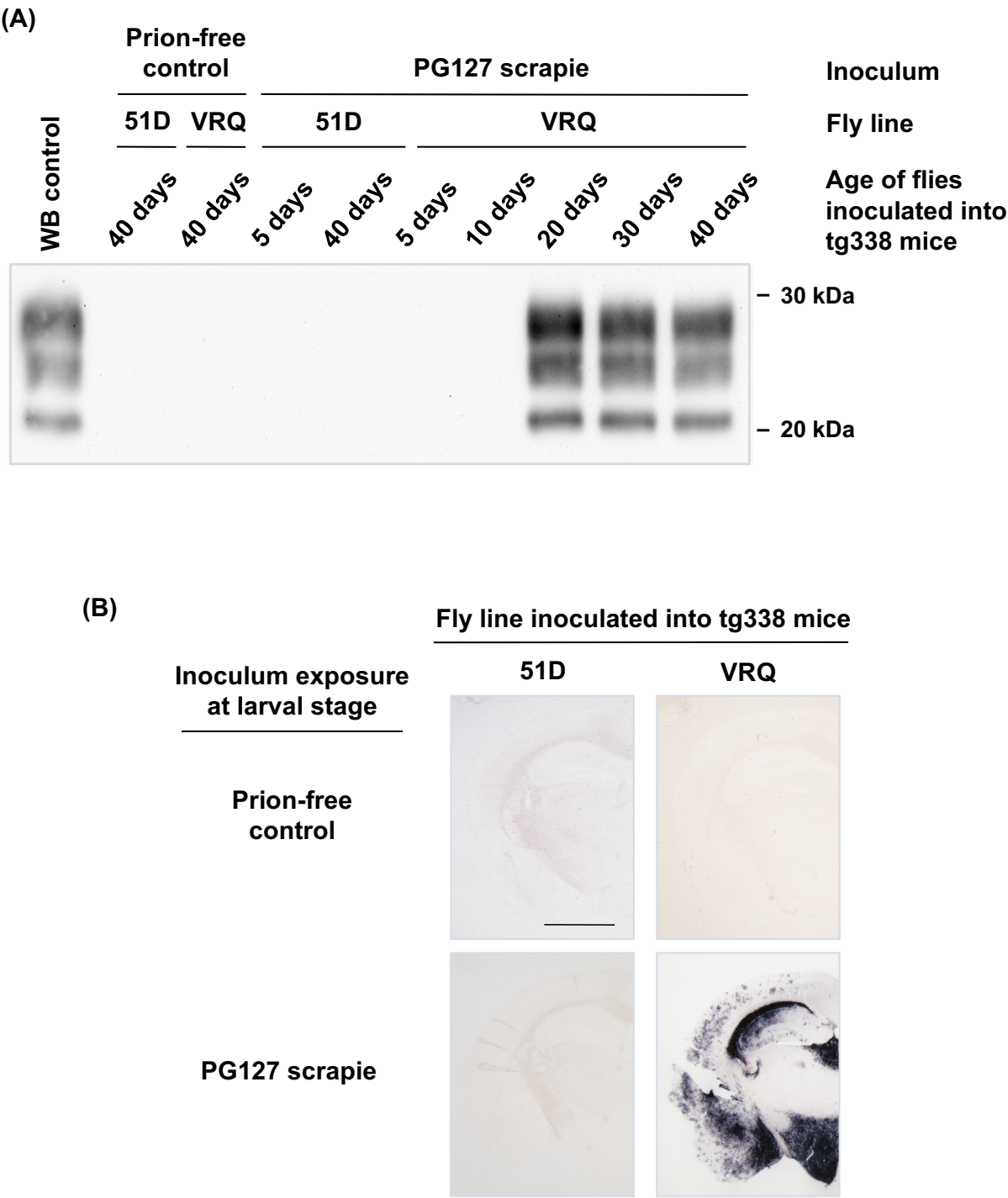
Figure 2. PK-resistant PrP in scrapie-exposed PrP transgenic *Drosophila*



**Table 1: Prion infectivity accumulates in scrapie-exposed PrP transgenic *Drosophila***

Fly line	Inoculum	Age of <i>Drosophila</i> inoculated into tg338 mice									
		5 days		10 days		20 days		30 days		40 days	
		Attack Rate	IP	Attack Rate	IP	Attack Rate	IP	Attack Rate	IP	Attack Rate	IP
51D	Control	0/6	>250	0/6	>250	0/6	>250	0/6	>250	0/6	>250
	PG127	0/6	>250	0/6	>250	0/6	>250	0/6	>250	0/6	>250
VRQ	Control	0/6	>250	0/6	>250	0/6	>250	0/6	>250	0/6	>250
	PG127	0/6	>250	0/6	>250	4/6	103 ± 11	6/6	89 ± 3	6/6	89 ± 2

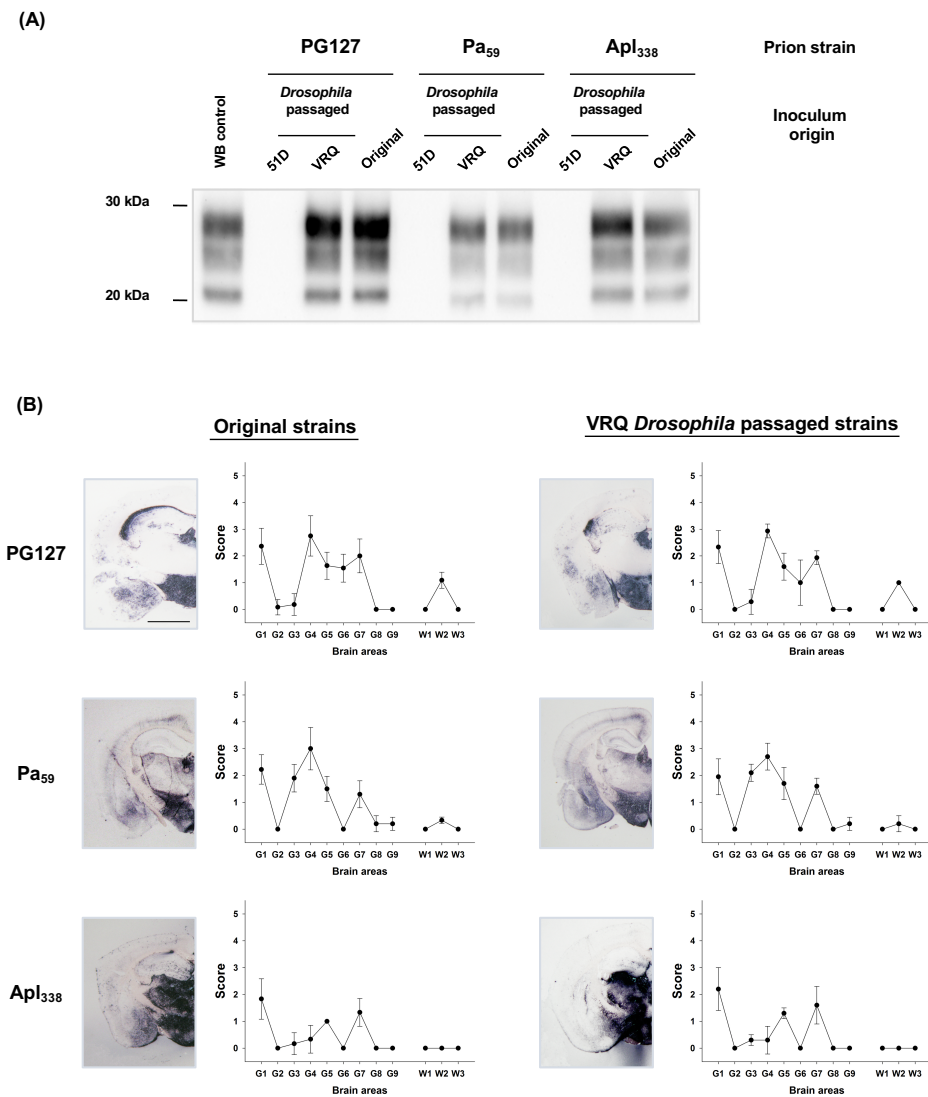
Figure 3. Detection of prion infectivity in scrapie-exposed PrP transgenic *Drosophila*



**Table 2: Scrapie strains retain transmission properties after passage in PrP transgenic *Drosophila***

Scrapie strain	Original strain isolated in tg338 mice		<i>Drosophila</i> -passaged strain isolated in tg338 mice			
			1 <sup>st</sup> Passage		2 <sup>nd</sup> Passage	
	Attack Rate	IP	Attack Rate	IP	Attack Rate	IP
PG127	6/6	61 ± 2	6/6	87 ± 4	6/6	59 ± 1
Pa <sub>59</sub>	6/6	143 ± 3	6/6	187 ± 4	6/6	142 ± 1
ApI <sub>338</sub>	6/6	631 ± 83	3/5	642 ± 57	NA	NA

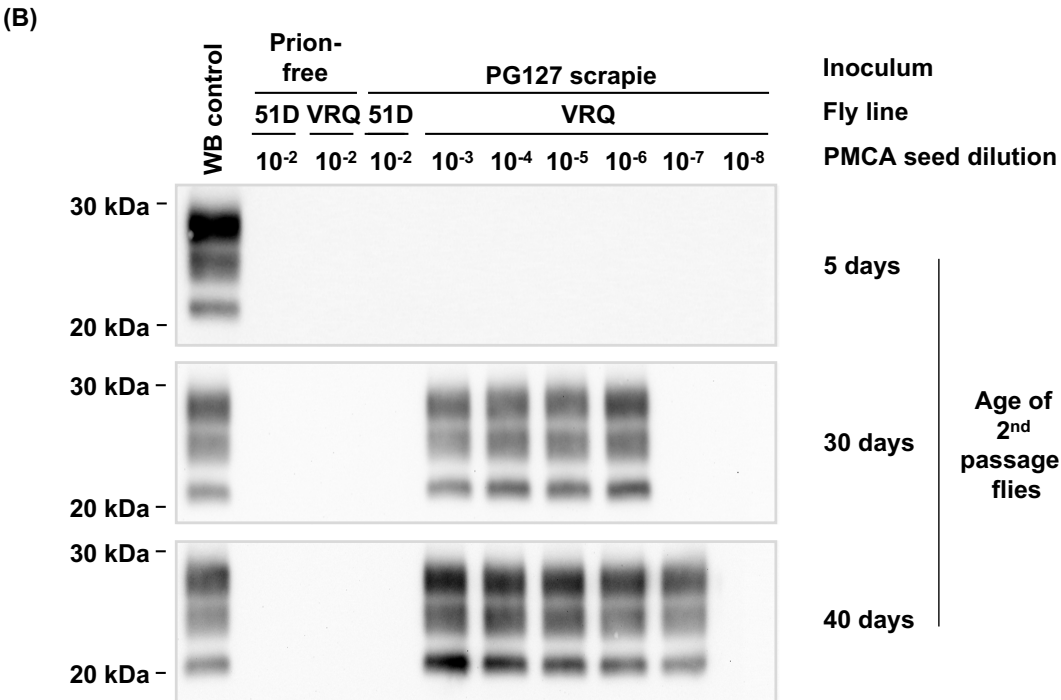
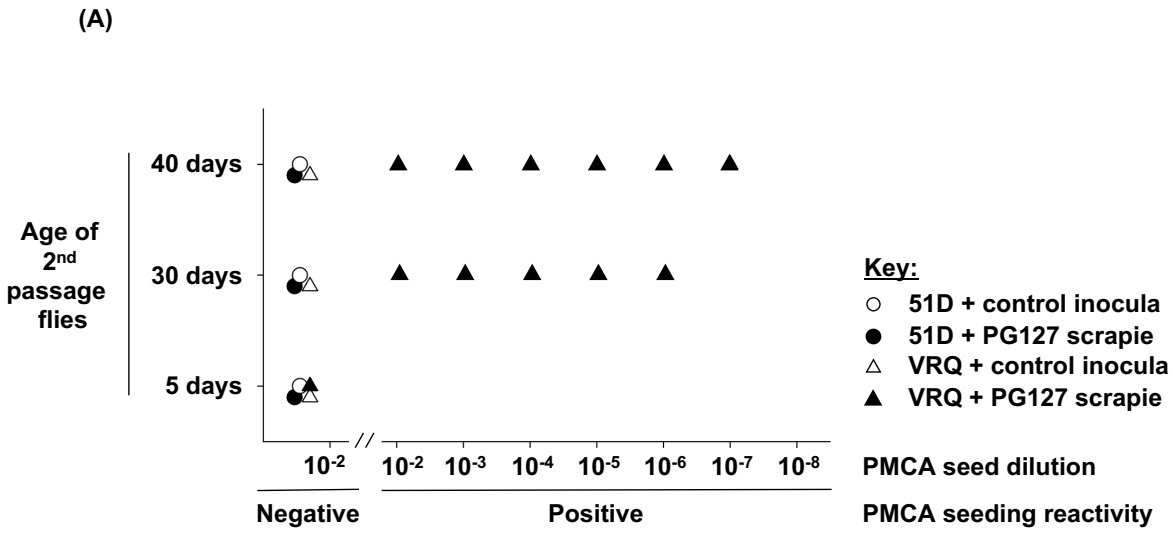
Figure 4. Authentic prion replication in PrP transgenic *Drosophila*



**Supplementary Data S1: Reproducible fly-to-mouse transmission of prions from scrapie-exposed PrP transgenic *Drosophila***

Experiment	Fly line	Inoculum	Transmission in tg338 mice	
			Attack Rate	IP
1	51D	Control	0/6	>670
		PG127	0/6	>670
	VRQ	Control	0/6	>250
		PG127	6/6	87 ± 4
2	51D	Control	0/6	>670
		PG127	0/6	>250
	VRQ	Control	0/6	>250
		PG127	6/6	87 ± 1
3	51D	Control	0/6	>250
		PG127	0/6	>250
	VRQ	Control	0/6	>250
		PG127	6/6	87 ± 2

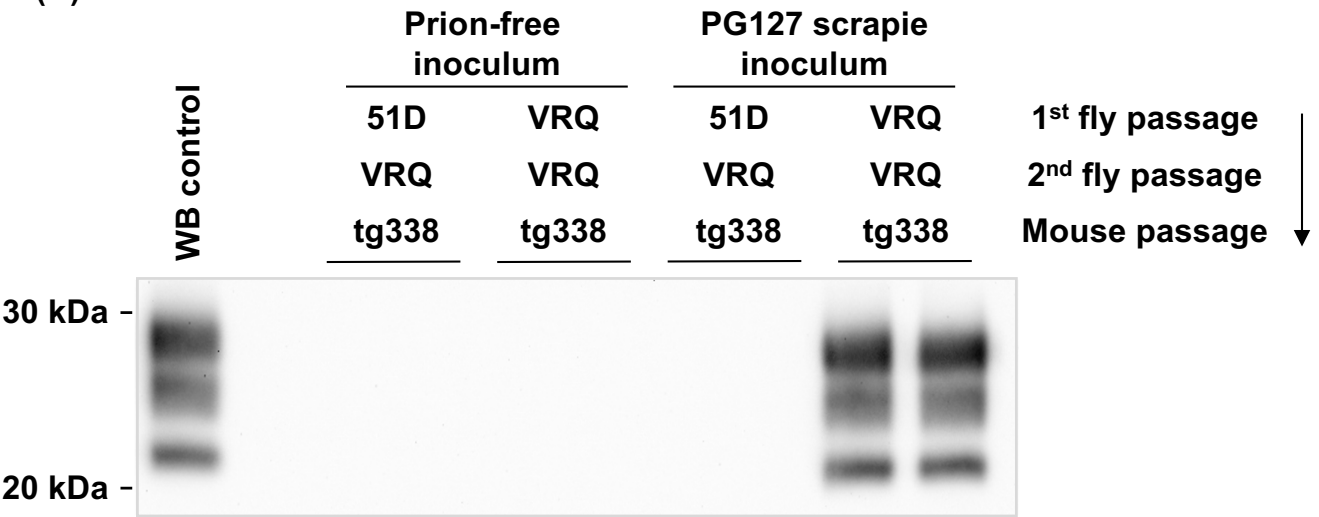
**Supplementary Data S2. Serial transmission of prion seeding activity in scrapie-exposed PrP transgenic *Drosophila***



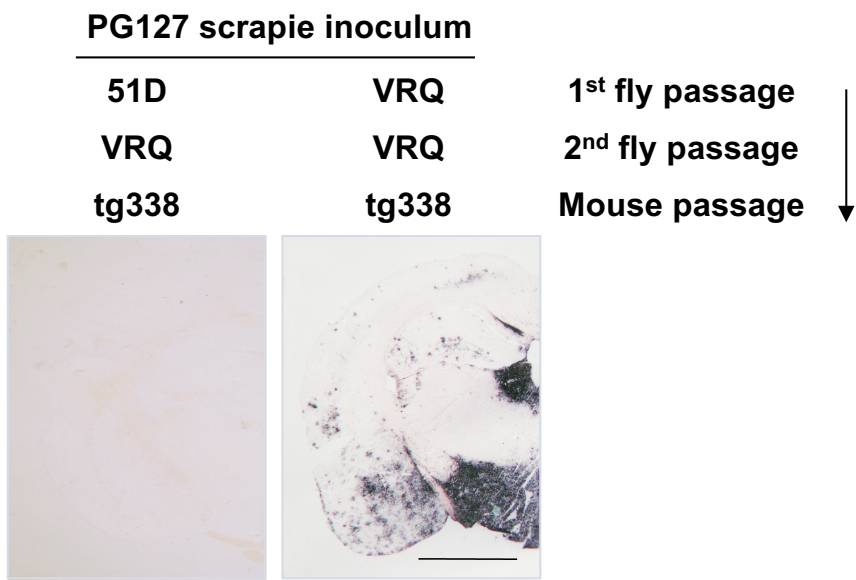


Supplementary Data S3. Serial transmission of prion infectivity in scrapie-exposed PrP transgenic *Drosophila*

(A)



(B)



Supplementary Data S4: Prion-induced toxicity in scrapie-exposed PrP transgenic *Drosophila*

